Slow softening of 'Kanzi' apples (*Malus* × *domestica* L.) is associated with preservation of pectin integrity in middle lamella

Sunny George Gwanpua [a,1] , Bert E. Verlinden [b], Maarten L.A.T.M. Hertog [a], Bart M. Nicolai [a,b], Marc Hendrickx [c], Annemie Geeraerd [a,*]

[a] KU Leuven, Division MeBioS, Department of Biosystems (BIOSYST), W. de Croylaan 42, bus 2428, B-3001 Leuven, Belgium

[b] Flanders Centre of Postharvest Technology, W. de Croylaan 42, B-3001 Leuven, Belgium

[c] KU Leuven, Laboratory of Food Technology and Leuven Food Science and Nutrition Research Centre (LFoRCe), Department of Microbial and Molecular Systems (M^2S), Kasteelpark Arenberg 22, PB 2457, B-3001 Leuven, Belgium

* Tel.: +32 163 20591; Fax: +32 163 21996; Email address: annemie.geeraerd@biw.kuleuven.be

Abstract

'Kanzi' is a recently developed apple cultivar that has extremely low ethylene production, and maintains its crispiness during ripening. To identify key determinants of the slow softening behaviour of 'Kanzi' apples, a comparative analysis of pectin biochemistry and tissue fracture pattern during different ripening stages of 'Kanzi' apples was performed against 'Golden Delicious', a rapid softening cultivar. While substantial pectin depolymerisation and solubilisation was observed during softening in 'Golden Delicious' apples, no depolymerisation or increased solubilisation was observed in 'Kanzi' apples. Moreover, tissue failure during ripening was mainly by cell breakage in 'Kanzi' apples and, in contrast, by cell separation in 'Golden Delicious' apples. 'Kanzi' apples had lower activity of beta-galactosidase, with no decline in the extent of branching of the pectin chain. A sudden decrease in firmness observed during senescence in 'Kanzi' apples was not due to middle lamella dissolution, as tissue failure still occurred by cell breakage.

[1] Current address: Centre for Postharvest and Refrigeration Research, Massey University, Palmerston North, New Zealand

Keywords: cell wall pectin, depolymerisation, softening, tissue failure, ethylene

1. Introduction

Softening in fleshy fruits is one of the main changes that occurs during ripening. Cell wall modifications and middle lamella polysaccharide dissolution are thought to be the main cause of flesh softening (Brummell & Harpster, 2001; Fischer, 1994; Giovannoni, 2001). Pectin polysaccharides play an important role in maintaining the integrity of the middle lamella, by acting as a crosslink between networks of xyloglucan-cellulose microfibrils (Carpita & Gibeaut, 1993). The crosslinking ability of pectin polysaccharides is dependent on its chemical structure, and modifications in pectin structure is largely responsible for softening during fruit ripening. The most common changes that occur in the cell wall pectin during ripening are debranching (due to losses in side chain neutral sugars of rhamnogalacturonan I, RG-I), depolymerisation, and solubilisation (Fischer, 1994; Gwanpua et al., 2014; Peña & Carpita, 2004). Additionally, in some fruits, there may be loosening of cell walls by expansin and xyloglucan endotransglucosylase/hydrolase (Atkinson, Johnston, Yauk, Sharma, & Schröder, 2009; McQueen-Mason & Cosgrove, 1995).

Several enzymes, acting in a concerted manner, are involved in the breakdown of cell wall pectin and loss of middle lamella integrity (Bennett & Labavitch, 2008; Brummell & Harpster, 2001; Goulao, Santos, de Sousa, & Oliveira, 2007). Side chain debranching enzymes, such as beta-galactosidase (β-GAL) and alpha-arabinofuranosidase (α-AF), and cell wall loosening expansins increase cell wall porosity, facilitating access of other enzymes. Pectin methylesterase (PME) removes the methyl group of the galacturonic acid residues of the pectin homogalacturonan backbone, while polygalacturonase (PG) depolymerises the demethylated pectin backbone.

'Kanzi' apple is a recently developed apple cultivar that is characterised by a firm texture and very low ethylene production (Gwanpua et al., 2013). It was developed in Belgium by a natural cross between 'Gala' and 'Braeburn' apples (Finn & Clark, 2008). 'Kanzi' apples can be kept at ambient shelf life conditions for several weeks without any noticeable loss in flesh firmness (Gwanpua et al., 2013). The current study aims at understanding the mechanistic basis of the ability of 'Kanzi' apples to remain crispy during ripening, by investigating the pectin biochemistry and tissue fracture pattern during ripening. Moreover, these cell wall properties

were simultaneously investigated during ripening of ‘Golden Delicious’ apples, a rapid softening apple cultivar, with relatively higher rates of ethylene production (Wakasa et al., 2006). The cell wall pectin polysaccharides of ‘Kanzi’ and ‘Golden Delicious’ apples at different ripening stages were characterised, the activities of different cell wall hydrolases were investigated, and the tissue fracture pattern was visualised by scanning electron microscopy. Key differences, with respect to middle lamella pectin biochemistry and tissue fracture pattern, between ‘Kanzi’ and ‘Golden Delicious’ apples will be discussed.

2. Materials and methods

2.1. Plant Material

‘Kanzi’ and ‘Golden Delicious’ (*Malus* × *domestica* Borkh.) apple fruits were harvested in 2014 from orchards in Flanders, Belgium. Fruit from each cultivar was harvested from the same row of trees within an orchard to limit batch-to-batch variability, which could obscure the cultivar differences. The apples were harvested at commercial maturity, using a combination of firmness (>73.6 N), starch (<7.5 based on the European starch chart), sugar and acid measurements (soluble solids content > 13%). Both cultivars were stored under optimal storage conditions for long term storage. For ‘Kanzi’, the fruit were stored at 4 °C under a controlled atmosphere (CA) condition of 2 % O_2 and 0.7 % CO_2, while the ‘Golden Delicious’ apples were stored at 1 °C under a CA condition of 2 % O_2 and 2.5 % CO_2. In addition, for the ‘Kanzi’ apples, the application of CA was delayed by 21 d after harvest according to commercial protocols, to avoid incidence of browning disorder. After 6 months, the apples were removed from storage and allowed to ripen under ambient shelf life conditions (18 °C under regular atmosphere, with relative humidity at 55 – 85 %) for 6 weeks. Apples coming out of storage were considered to be at the ‘onset’ of ripening, while those that had additionally been kept for two and six weeks under ambient ripening conditions will be denoted as ‘ripe’ and ‘senescent’ respectively.

2.2. Physiological assessment

Tissue biomechanical properties (firmness, yield stress, and yield strain) were measured using an LRX Universal Testing Machine (Lloyd Instruments, UK) (Harker, Stec, Hallett, & Bennett, 1997). The firmness was measured by driving a self-cutting cylindrical plunger with a surface of

1 cm^2, attached to a load cell of 500 N, at a constant speed of 8 mm s^{-1} into the fruit. The firmness was taken as the maximum force (N) needed for the plunger to penetrate the fruit to a depth of 8 mm. Two measurements were taken on the equator, 180° apart, and the average was taken as the firmness value. For 'Kanzi' apples, these two measurements were taken at the blush side and at the green side. Measurements were done with the fruit skin intact. For each sampling point, the firmness of 10 fruit was measured.

The yield stress and yield strain of the same fruit were measured using a tissue cylinder (16 mm in diameter, 20 mm in height), excised from the apple outer cortex, close to the region where the penetrometer readings were taken. Two 10 mm diameter cork borers, separated by a distance of 4 mm, were used to create notches at each side midway of the tissue cylinder, providing a weakened zone. The tissue block was glued at both ends to PVC materials and attached to the LRX Universal Testing Machine, and pulled apart at a constant speed of 10 mm min^{-1} until tissue failure. The yield stress was calculated as the ratio of the tensile strength (maximum force before tissue failure) to the cross sectional area of the weakened zone, while the yield strain (strain at failure) was calculated as the maximum deformation relative to the original length of the weakened zone (10 mm). Fractured tissues were stored in 70 % ethanol, and the fractured surfaces were viewed in a scanning electron microscope to visualise cell failure, as will be detailed in section 2.8.

Ethylene production was measured according to the protocol of Bulens et al. (2011). An apple was enclosed in a jar of 1.1 L and flushed for 1 hour with humidified air at 18 °C. The inlet and outlet were then closed and the jars were kept at 18 °C for 4 h to allow for sufficient ethylene accumulation. 3 mL gas samples were withdrawn from the jars and analysed by injecting into a CompactGC (Interscience, Louvain-la-Neuve, Belgium). Calibration was done by ethylene standards ranging from 50 μg L^{-1} to 50 mg L^{-1}. For each sampling time, the ethylene production of six fruit was measured individually.

Also, the weight of 20 fruit were individually monitored, to estimate moisture loss during ripening.

2.3. Cell wall pectin extraction and fractionation

Cell wall materials were isolated using a method adapted from Renard (2005). The absence of starch was confirmed by performing a starch iodine test on apples at the 'onset' stage (Supplementary Figure S1). About 30 g of apple cortex tissue was chopped into small pieces and dropped into a boiling solution of 70 % (v/v) ethanol for 20 min, to inactivate endogenous enzymes. The sample was then frozen in liquid nitrogen, crushed, and homogenised in 200 mL 95 % (v/v) ethanol. The suspension was filtered (Macherey-Nagel MN 615 Ø 90 mm, Germany) and the residue was again homogenised in 100 mL 95 % (v/v) ethanol. Following filtration, the residue was homogenised in 100 mL acetone. The final residue was dried overnight at 40 °C to obtain the alcohol insoluble residue (AIR). The cold water extractable (WEP), chelator extractable (CEP), and Na_2CO_3 extractable (NEP) pectin fractions were sequentially extracted from the AIR. WEP was extracted by stirring 0.25 g of AIR in 50 mL of distilled water for 6 h at 25 °C. Extraction of CEP and NEP were done following the protocol described in Gwanpua et al., (2014). Basically, CEP was obtained by stirring the residue resulting from WEP extraction in a solution of 0.05 M cyclohexane-trans-1,2-diamine tetra-acetic acid for 6 h, while NEP was subsequently extracted from the resulting residue by incubating in a solution of 0.05 M Na_2CO_3, containing 0.02 M $NaBH_4$, for 22 h. The uronic acid content of the AIR and the different pectin fractions was measured spectrophotometrically at 520 nm, following complete hydrolysis by concentrated sulphuric acid (Blumenkrantz & Asboe-Hansen, 1973). For each ripening stage, the AIR was extracted and fractionated from four individual fruit (biological replicates). Lyophilised samples of the different pectin fractions were obtained by freeze drying, following dialysis for 48 h (molecular weight cut-off of 12–14 kDa) against demineralized water to minimize the presence of small co-solutes. For the CEP fraction, there was initial dialysis against 0.1 M NaCl for 24 h followed by dialysis against demineralised water for another 24 h. The cell wall pectins of these lyophilised samples were further characterised as described in sections 2.4 – 2.6.

2.4. Determination of degree of methylesterification

The pectin degree of methylesterification (DM) was determined using FT-IR spectroscopy, as described by Kyomugasho, Christiaens, Shpigelman, Van Loey, & Hendrickx (2015). The pH of the samples was confirmed to be around 6.0. A sample from the dry material (AIR or lyophilised

WEP) was firmly compacted to expel entrapped air and create smooth surfaces. The sample was then placed on the sample holder of the FT-IR (Shimadzu FTIR-8400S, Japan), and the transmittance was recorded at wavenumbers from 4000 cm^{-1} to 400 cm^{-1}, at a resolution of 4 cm^{-1}. For each sample, the spectra of 100 scans were averaged to ensure a high signal to noise ratio. The spectra were converted into absorbance mode prior to base line correction and reading of the absorption at the maxima of both peaks. The obtained ratio (R) between the intensity of the peak situated at 1740 cm^{-1} (due to the ester carbonyl group (C═O) stretching) to the combined intensities of the peak at 1740 cm^{-1} and the peak at 1600 cm^{-1} (due to carboxylate group (COO^-) stretching) (Szymanska-Chargot & Zdunek, 2013) was used to calculate the DM based on the calibration line $DM = 136.86 \times R + 3.987$ (Kyomugasho et al., 2015).

2.5. Neutral sugar analysis

The neutral sugar content of the different pectin fractions was quantified by a high-performance anion exchange chromatography (HPAEC) using a Dionex system (DX600), equipped with a GS50 gradient pump, a CarboPac™ PA20 column, a CarboPac™ PA20 guard column, and an ED50 electrochemical detector (Dionex, Sunnyvale, USA). The lyophilised samples (0.005 g) of the pectin fractions were hydrolysed in 0.5 mL of 4 M trifluoroacetic acid (TFA) at 110 °C for 1.5 h. The digested samples were dried under N_2 evaporator at 45 °C, and the TFA was removed by adding 1 M NH_4OH and drying again under N_2 at 45 °C. The dried sample was dissolved in ultrapure water (organic free, 18.2 MΩ cm resistance), filtered through a 0.45 μm filter (Chromafil® A-45/25, Germany) and injected onto the HPAEC system. Prior to sample injection, the system was equilibrated for 5 min using 100 mM NaOH, and for additional 5 min using 4 mM NaOH. Samples (10 μL) were injected and eluted for 20 min at a flow rate of 0.5 mL/min with 4 mM NaOH at 30 °C, followed by column regeneration (for 10 min) using 500 mM NaOH. Commercial neutral sugar standards at varying concentrations (1-10 mg L^{-1}) were used as external standards for identification and quantification. To correct for degradation of the monosaccharides during the acid hydrolysis step, mixtures of the sugar standards were subjected to the aforementioned hydrolysis conditions, and the peak areas were compared to those of untreated standard mixtures (Houben, Jolie, Fraeye, Van Loey, & Hendrickx, 2011).

2.6. Analysis of molecular mass distribution

The molecular mass distribution of the different WEP fractions was investigated using size exclusion chromatography. We focused on the WEP fractions as they are easily accessible by polygalacturonase and should contain the shorter pectin fragments (increased solubilisation) due to the activity of this enzyme. In addition, this fraction is less sensitive to pectin aggregation (due to cross linking) during the HPLC SEC measurements and the sample pre-filtering. The separation was performed on series of three Waters columns (Waters, Milford, MA), namely, Ultrahydrogel 250, 1000, and 2000 with exclusion limits of 8×10^4, 4×10^6, and 1×10^7 g mol^{-1}, respectively. The eluent was monitored using a reflective index detector (Shodex RI-101, Showa Denko K.K., Kawazaki, Japan). A 100 µL of 0.5 % of the lyophilised WEP fraction was solubilized in the eluent and filtered through 0.45 µm filter before being injected by an autosampler (G1329A, Agilent technologies, Diegem, Belgium). The eluent (0.1 M MES (2-(N-morpholino) ethanesulfonic acid buffer, pH 6.5), with 0.1 M NaCl to assure a stable pH and minimize ionic interactions) was prepared using ultrapure water (organic free, 18 MΩ cm resistance), filtered (0.1 µm) and degassed by an on-line degasser of the HPLC system (Agilent technologies 1200 Series, Diegem, Belgium). The flow rate was 0.5 mL min^{-1} and the columns were kept at 35 °C.

2.7. Extraction and assay of PME, β-Gal, and α-AF enzymes

PME, β-Gal, and α-AF enzymes were extracted and assayed from crushed frozen apple samples at 'onset', 'ripe', and 'senescent' stages as defined above, following the protocols used by Gwanpua et al. (2014). PME was extracted using 0.2 M Tris buffer pH 7.5, containing 1.0 M NaCl, 0.1 % (v/v) Triton X-100, and 5 % Polyvinylpolypyrrolidone (PVPP); β-Gal was extracted using 10 mM borate buffer pH 9.0, containing 1.0 M NaCl, 0.1 % (v/v) Triton X-100, and 5 % PVPP, and α-AF was extracted using a 20 mM borate buffer pH 9.0, containing 2.0 M NaCl, 0.2 % (v/v) Triton-100, 5 % PVPP, 3 mM $ZnCl_2$ and 2 % (w/v) sucrose. All extractions were done for 2 h at 4 °C.

PME activity was assayed using the method described by Ly-Nguyen, Van Loey, Fachin, Verlent, & Hendrickx (2002). 0.2 mL of enzyme extract was added to a solution of 0.35 % apple pectin, containing 0.117 M NaCl. The reaction mixture was incubated at 22 °C, while titrating with 0.01 M NaOH to maintain the pH at 7.5 using an automatic pH-stat (718 STAT titrino, Ω

Metrohm, Switzerland). One unit of PME activity was defined as the amount of enzyme required to release 1 μmol of carboxyl group per min, under the assay conditions.

β-Gal was assayed as described in Tateishi, Inoue, & Yamaki (2001). The reaction mixture containing 0.2 mL of the enzyme extract, 0.5 mL of 0.1 M citrate buffer (pH 4.0), 0.1 mL BSA solution and 0.4 mL of 13 mM p-nitro phenyl-β-D-galactopyranoside was incubated for 10 min at 37 °C. The reaction was stopped by adding 2 mL of 0.2 M Na_2CO_3 solution and the absorbance at 400 nm was measured. One unit of enzyme activity was defined as the amount of enzyme that hydrolyses the release of 1 μmol of p–nitrophenol per min, under the given assay conditions.

α-AF were assayed using the method used by Tateishi, Kanayama, & Yamaki (1996). 0.1 mL of the enzyme extract was incubated in a reaction mixture consisting of 0.25 mL of 0.1 M citrate buffer (pH 4.0), 0.05 mL BSA solution, and 0.2 mL of 3.6 mM p-nitrophenyl-α-L-arabinofuranoside, at 37 °C. The reaction was stopped after incubation for 10 min by adding 2 mL of 0.2 M Na_2CO_3 solution, and the absorbance was measured at 400 nm. One unit of enzyme activity was defined as the amount of enzyme that hydrolyses the release of 1 μmol of p–nitrophenol per min, under the given assay conditions.

The enzyme activities were measured in fruit at each ripening stage, using samples from the same four biological replicates used for characterising cell wall pectin.

2.8. Scanning electron microscopy

Sample preparation was done following the protocol used by Vrijdaghs, Flores-Olvera, & Smets (2014). The fractured surfaces of the apple tissues were prepared for critical point drying by washing twice with 70 % ethanol for 5 min. Next, the material was placed in a mixture (1:1) of 70 % ethanol and dimethoxymethane (DMM) for 5 min, and subsequently transferred to 100 % DMM for 20 min. Critical point drying was done using liquid CO_2, with a CPD 030 critical point dryer (BAL-TEC AG, Balzers, Liechtenstein). The dried samples were mounted on aluminium stubs using Leit-C and coated with gold via a SPI-ModuleTM Sputter Coater (SPI Supplies, West-Chester, PA, USA). Scanning electron microscope (SEM) images were obtained with a Jeol JSM-6360 (JEOL Ltd., Tokyo) at the Ecology, Evolution and Biodiversity Conservation

Section (KU Leuven). Fracture surfaces of tissue samples from three different apples at each ripening stage were observed to obtain representative images.

2.9. Data analysis

Significant differences ($p < 0.05$) between means were investigated using one-way ANOVA. A multiple comparison was carried out between all factor level combinations using Tukey's Honest Significant Difference (HSD) test using the Statistics Toolbox of Matlab (The Mathworks Inc., Natick, USA). For each variable analysed, significant differences were estimated in common for all means within both cultivars, for all ripening stages.

3. Results

3.1. Climacteric and post-climacteric evolution of firmness, ethylene production, and moisture loss in 'Kanzi' and 'Golden Delicious' apples

'Kanzi' apples remained firm up to four weeks at 18 °C under normal atmosphere. However, between the fourth and sixth week of ripening, there was a sudden drop in firmness (Fig. 1A). This sudden decline in firmness coincided with considerable visible shrinkage. The 'Golden Delicious' apples were much softer than the 'Kanzi' apples already at the beginning of the shelf life exposure, and underwent further loss in firmness during ripening under ambient shelf life conditions (insert Fig 1A). There was also a sudden decline in firmness between the fourth and sixth week of shelf life in 'Golden Delicious', but this was less drastic than those observed for 'Kanzi' apples. The 'Kanzi' apples had extremely low ethylene production throughout ripening (Fig. 1B), several hundred folds less than 'Golden Delicious'. Both cultivars had peak ethylene production one week after exposure to ambient shelf life conditions. 'Golden Delicious' apples were able to maintain this high level of ethylene production until the fourth week of ripening, after which ethylene production started to decline. Ethylene production in 'Kanzi' apples declined immediately after reaching the – relatively very low – climacteric maximum. Both cultivars had comparable moisture loss, which increased linearly with time to about 10 % after 6 weeks of shelf life (Fig. 1C) resulting in visually shrivelled fruit at 'senescent'.

3.2. Changes in pectin fractions and depolymerisation of WEP

The content of the different pectin fractions during the different ripening stages for both 'Kanzi' and 'Golden Delicious' apples is shown in Figure 2A. There was no change in the amount of WEP, CEP, and NEP in 'Kanzi' for all three ripening stages. For 'Golden Delicious' apples, only the WEP fraction changed during ripening, with a steady increase observed from the 'onset' to the 'ripe' and 'senescent' stages. Consequently, the size exclusion chromatographic (SEC) profile of the WEP fraction should provide information about possible pectin depolymerisation. The SEC profile of the WEP for both 'Kanzi' and 'Golden Delicious' apples during the different ripening stages is shown in Figure 2B. A clear shift from large size pectin at the 'onset' stage to smaller size pectin at the 'ripe' and 'senescent' stages was observed in 'Golden Delicious' apples. Pectin depolymerisation in 'Golden Delicious' was more evident between the 'onset' and 'ripe' stages, with very little changes occurred between the 'ripe' and the 'senescent' stages. Contrarily, 'Kanzi' apples ripen without any noticeable pectin depolymerisation, based on the SEC profiles of the WEP fractions. In fact, there was a shift in the molar mass distributions to larger size pectin between the 'onset' and the 'ripe' stages (Fig. 2B).

3.3. Loss of pectin neutral sugars, and activities of side-chain debranching enzymes beta-galactosidase and alpha-arabinofuranosidase

The galactose and arabinose content of different pectin fractions were quantified for both 'Kanzi' and 'Golden Delicious' apples at the 'onset', 'ripe', and 'senescent' stages (Fig. 3A). The galactose content of the WEP fraction was higher in 'Kanzi' apples than in 'Golden Delicious' during the 'ripe' stage. Also, the amount of galactose and arabinose declined between the 'onset' and 'ripe' stages in 'Golden Delicious' apples, but not in 'Kanzi'. No significant differences were observed between the galactose and arabinose content in the CEP fractions for both apple cultivars, and no changes were observed during the different ripening stages. The NEP fractions were richer in arabinose, with higher values found in 'Golden Delicious'. No clear decline in galactose or arabinose content was observed in the NEP fractions for either cultivar. To get an idea of the extent of branching of the pectin side chain, the ratio of the sum of galactose and arabinose contents to the rhamnose content was calculated for all three pectin fractions (Fig 3B). The extent of branching declined between the 'onset' and 'ripe' stages for 'Golden Delicious' apples in both the WEP and NEP fractions. The activities of both beta-galactosidase (β-GAL)

and alpha-arabinofuranosidase (α-AF) were much higher in 'Golden Delicious' apples (Fig 3C). A high β-GAL activity was observed between the 'onset' and 'ripe' stages for 'Golden Delicious' apples, but declined between the 'ripe' and 'senescent' stages. α-AF activity was much lower, and not significantly different between both cultivars. Moreover, the α-AF activity did not change significantly during any of the ripening stages.

3.4. Pectin degree of methylesterification and pectin methylesterase activity

The degree of methylesterification (DM) of the alcohol insoluble residues (AIR) was comparable for the two cultivars, and constant throughout ripening (Fig. 4A). The WEP fractions of 'Kanzi' apples had lower DM values (between 50 and 60 %) than those of 'Golden Delicious' (Fig. 4B), but remained constant throughout ripening. The pectin methylesterase (PME) activity was not significantly different between the 'Golden Delicious' and 'Kanzi' apples, and decreased drastically between the 'ripe' and 'senescent' stage to undetectable levels in both cultivars (Fig. 4C)

3.5. Fracture pattern of 'Kanzi' and 'Golden Delicious' apples during ripening

The fractured surface observed by SEM revealed differences in the mode of tissue failure in 'Kanzi' and 'Golden Delicious' apples. At all ripening stages, tissue failure in 'Kanzi' was almost exclusively by cell breakage (Fig. 5A-C). For 'Kanzi' apples at the 'onset' stage, the ruptured surfaces were regularly shaped, with high yield stress (Fig. 6A), and low yield strain (Fig. 6B). During the 'ripe' and 'senescent' stages, tissue failure was still by cell breakage, with low tensile stress but large tissue deformation (Fig. 6A & B). Tissue failure in 'Golden Delicious' apples at the 'onset' stage was both by cell breakage and cell separation, as there were both ruptured and non-ruptured cells. However, as ripening progressed, tissue failure was almost exclusively by cell separation. Unlike in 'Kanzi' apples, there was no increase in tissue deformation (yield strain) in 'Golden Delicious' apples at the 'senescent' stage (Fig. 6B).

4. Discussion

4.1. ‘Kanzi’ apples ripen with minimal modifications in cell wall pectin

By comparing softening-related cell wall modifications and tissue fracture patterns of ‘Kanzi’ apples and the rapid softening ‘Golden Delicious’, important determinants of softening were identified. Several studies on apple softening have suggested that the loss in side chain galactose and arabinose from the RG-I side chain of the cell wall pectin, concomitant with increased activities of β-GAL and α-AF, occurs during softening (Gwanpua et al., 2014; Ng et al., 2015; Peña & Carpita, 2004). In the current study, the activity of β-GAL was higher in ‘Golden Delicious’ than in ‘Kanzi’ apples. α-AF activity remained constant during climacteric ripening, but appeared to increase during the post-climacteric ripening in both cultivars. This is in accordance with an earlier study where loss in arabinose neutral sugars was shown to occur during the later stages of ripening in different apple cultivars (Peña & Carpita, 2004). However, in this study there was no significant decrease in the arabinose content during any of the ripening stages, in any of the pectin fractions for both ‘Golden Delicious’ and ‘Kanzi’ apples. Moreover, the high β-GAL activity during climacteric ripening in ‘Golden Delicious’ apple seems not to be accompanied by any significant loss in side chain galactose in any of the pectin fractions. Nevertheless, by considering the extent of side chain branching, rather than the absolute arabinose and galactose content, there appears to be more side chain neutral sugar degradation in ‘Golden Delicious’ apples. This decline in extent of branching was most evident in the WEP and NEP fractions between the ‘onset’ and ‘ripe’ stages, suggesting that there is little pectin side-chain neutral sugar degradation during senescence in ‘Golden Delicious’ apples. This was concomitant to the decline in β-GAL activity in ‘Golden Delicious’ between the ‘ripe’ and ‘senescent’ stages.

Pectin depolymerisation and increased solubilisation were more obvious softening-dependent cell wall pectin modifications. The complete absence of any measurable increase in solubilisation or depolymerisation of the WEP fraction in ‘Kanzi’ could be the main reason for the lack of detectable loss in firmness during normal climacteric ripening. An apparent shift in the SEC profile of the WEP towards pectin with higher molecular weight observed in ‘Kanzi’ could be related to the extraction of WEP fractions with larger sizes in latter ripening stages. Also, it could be due to possible aggregation of WEP pectin polysaccharides via Ca^{2+}

crosslinking, particularly as the DM of the WEP fraction of ‘Kanzi’ apples were lower than those of ‘Golden Delicious’ apples. An earlier study on evolution of firmness in ‘Kanzi’ also showed an increase in firmness during ripening (Gwanpua et al., 2013). Pectin depolymerisation and solubilisation was clearly evident in the rapid softening ‘Golden Delicious’ apples. Although in several studies endo-PG activity was undetectable in ripe apples (Goulao et al., 2007; Yoshioka, Aoba, & Kashimura, 1992), the *endo-polygalacturonase 1* (*PG1*) gene has been shown to be strongly associated with apple fruit softening (Atkinson et al., 2012; Gwanpua et al., 2016; Longhi et al., 2013). The close connection between depolymerisation of pectin and softening in ‘Golden Delicious’ apples supports the earlier reported role of *PG1* in softening of ‘Golden Delicious’ apple (Longhi et al., 2013). The lack of any measureable increase in pectin solubilisation and depolymerisation during ripening in ‘Kanzi’ apples is in line with the observed firmness and tissue failure behaviour. Tissue failure in ‘Kanzi’ apples was mainly by cell breakage at all ripening stages, parallel to the absence of any noticeable increase in pectin depolymerisation and solubilisation.

Another important observation from this study was that the overall DM of the cell wall pectin (or its fractions) does not necessarily need to be very low for depolymerisation to occur. The DM of WEP fraction in ‘Golden Delicious’ apples were higher than 70 %, suggesting a rather limited sensitivity of pectin to PG induced degradation. However, most plant PMEs are processive enzymes, such that de-esterification occurs in blocks, providing regions of no methyl esterification for depolymerisation (Catoire, 1998; Markovič & Kohn, 1984). The pectin DM on itself does not provide a good indicator for softening, nor does the activity of PME during ripening. PME activity was not higher in the ‘ripe’ ‘Kanzi’ than in the ‘ripe’ ‘Golden Delicious’, yet the former had a lower DM for the WEP fractions. Elsewhere, Ng et al. (2013) did not observe any significant differences in PME activity or degree of esterification between ‘Scifresh’ (slow softening) and ‘Royal Gala’ (rapid softening) apple cultivars at the ‘mature’ and ‘ripe’ stages, further suggesting that PME is unlikely to be a key determinant in differences in softening behaviour amongst different apple cultivars.

4.2. Post-climacteric softening in ‘Kanzi’ apples is driven by possible cell collapse from excessive dehydration

An interesting observation in the current study was the drastic decline in firmness of ‘Kanzi’ apples between the ‘ripe’ and ‘senescent’ stages in the absence of any measurable increase in

pectin depolymerisation or solubilisation (nor increase of WEP pectin fraction, nor changes in the WEP molar mass distribution pattern). This suggests that factors other than increased cell wall dissolution were responsible for softening during post-climacteric ripening in the 'Kanzi' apples. Saladié et al. (2007) showed that transpirational water loss could be as integral in softening as is cell wall disassembly. Moisture loss during ripening may directly influence firmness by loss of cell turgor (Barrett, Garcia, & Wayne, 1998; Fanta et al., 2014; Jackman, Marangoni, & Stanley, 1992). While tissue failure amongst all ripening stages in 'Kanzi' was mainly by cell breakage, the sudden decline in firmness between the 'ripe' and 'senescent' stages was accompanied by a drastic increase in the yield strain (tissue deformation), with the tissue behaving as a ductile material. A possible explanation to this observation is that at the 'onset' stage the cellular turgor was very high, but the cells became flaccid as the fruit entered the post-climacteric stage, due to excessive dehydration to levels far beyond the turgor-loss point. It would be interesting to see if the sudden decline in the firmness of 'Kanzi' apples coincide with a sudden loss in cell turgor. In a study by Tong et al. (1999), the crispy texture of 'Honeycrisp' was shown to be related to its ability to maintain high turgor potential and cell wall integrity. Also, the fracture surfaces of ripe tissues of 'Scifresh' (a slow softening apple cultivar) was shown to have greater cell rupture and greater cell adhesion that the rapid softening 'Royal Gala' (Ng et al., 2013).

In 'Golden Delicious' apples where tissue failure in the 'ripe' and 'senescent' fruit occurred mainly by cell separation, the separated cells were irregularly shaped, suggesting that a combination of loss in cell turgor and dissolution of the pectin in the middle lamella could have been responsible for softening. Surprisingly, the moisture loss for both apple cultivars were comparable. This implies that the differential softening behaviour of 'Kanzi' and 'Golden' was not directly linked to differences in transpirational water loss, but more to the fact that water loss in 'Kanzi' was not accompanied by substantial pectin breakdown. Unlike in 'Kanzi', 'there was no increase in tissue yield strain between the 'ripe' and 'senescent' stages for 'Golden Delicious' apples, as moisture loss was accompanied by significant solubilisation and depolymerisation of the pectin in the middle lamella. This also explains why the tissue yield stress was much higher for 'Kanzi' than for 'Golden Delicious' apples.

5. Conclusion

The cell wall pectin changes and the tissue fracture pattern of 'Kanzi', an apple cultivar with a low ethylene production and ability to remain crisp during ripening, was investigated. The limited increase in cell wall polymer solubilisation, as revealed by the limited increase of pectin depolymerisation and solubilisation, was associated with a slow softening behaviour. In addition, tissue failure in 'Kanzi' apples was mainly by cell breakage, even in the post-climacteric state, suggesting the integrity of pectin in the middle lamella was maintained during ripening. Even under conditions of limited increase of cell wall pectin dissolution, apple can undergo loss in firmness after extensive dehydration. The high level of pectin depolymerisation that was accompanied by solubilisation and softening during ripening in 'Golden Delicious' apples are in line with the key role of depolymerising enzymes, such as endo-polygalacturonase, in softening of certain apple cultivars.

6. Acknowledgments

This publication has been produced with the financial support of the European COST Action FA1106 ('QualityFruit'), the Research Council of the KU Leuven (OT 12/055, postdoctoral fellowship of author SGG) and the Flanders Fund for Scientific Research (project G.0603.08). The opinions expressed in this document do not by any means reflect the official opinion of the European Union or its representatives.

7. References

Atkinson, R. G., Johnston, S. L., Yauk, Y. K., Sharma, N. N., & Schröder, R. (2009). Analysis of xyloglucan endotransglucosylase/hydrolase (XTH) gene families in kiwifruit and apple. *Postharvest Biology and Technology*, *51*(2), 149–157.

Atkinson, R. G., Sutherland, P. W., Johnston, S. L., Gunaseelan, K., Hallett, I. C., Mitra, D., … Schaffer, R. J. (2012). Down-regulation of *POLYGALACTURONASE 1* alters firmness, tensile strength and water loss in apple (*Malus* x *domestica*) fruit. *BMC Plant Biology*. http://doi.org/10.1186/1471-2229-12-129

Barrett, D. M., Garcia, E., & Wayne, J. E. (1998). Textural modification of processing tomatoes. *Critical Reviews in Food Science and Nutrition*, *38*(3), 173–258. http://doi.org/10.1080/10408699891274192

Bennett, A. B., & Labavitch, J. M. (2008). Ethylene and ripening-regulated expression and function of fruit cell wall modifying proteins. *Plant Science*, *175*(1-2), 130–136. http://doi.org/10.1016/j.plantsci.2008.03.004

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry*, *54*(2), 484–489. http://doi.org/10.1016/0003-2697(73)90377-1

Brummell, D. A., & Harpster, M. H. (2001). Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. *Plant Molecular Biology*, *47*(1-2), 311–339. http://doi.org/10.1023/A:1010656104304

Bulens, I., Van de Poel, B., Hertog, M. L., De Proft, M. P., Geeraerd, A. H., & Nicolaï, B. M. (2011). Protocol: An updated integrated methodology for analysis of metabolites and enzyme activities of ethylene biosynthesis. *Plant Methods*, *7*(1), 17. http://doi.org/10.1186/1746-4811-7-17

Carpita, N. C., & Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal*, *3*(1), 1–30. http://doi.org/10.1111/j.1365-313X.1993.tb00007.x

Catoire, L. (1998). Investigation of the action patterns of pectinmethylesterase isoforms through kinetic analyses and NMR Spectroscopy. implications in cell wall expansion. *Journal of Biological Chemistry*, *273*(50), 33150–33156. http://doi.org/10.1074/jbc.273.50.33150

Fanta, S. W., Abera, M. K., Aregawi, W. A., Ho, Q. T., Verboven, P., Carmeliet, J., & Nicolai, B. M. (2014). Microscale modeling of coupled water transport and mechanical deformation of fruit tissue during dehydration. *Journal of Food Engineering*, *124*, 86–96. http://doi.org/10.1016/j.jfoodeng.2013.10.007

Finn, C. E., & Clark, J. R. (2008). Register of new fruit and nut cultivars list 44. *Hortscience*, *43*(5), 1321–1343. Retrieved from http://apps.webofknowledge.com/full_record.do?product=UA&search_mode=GeneralSearch&qid=1&SID=W2vApbnOkZNr52TjgLt&page=2&doc=18&cacheurlFromRightClick=no

Fischer, M. (1994). Changes in the pectic substances of apples during development and postharvest ripening. Part 2: Analysis of the pectic fractions. *Carbohydrate Polymers*, *25*(3), 167–175. http://doi.org/10.1016/0144-8617(94)90201-1

Giovannoni, J. (2001). Molecular biology of fruit maturation and ripening . *Annual Review of Plant Physiology and Plant Molecular Biology*, *52*, 725–749. http://doi.org/10.1146/annurev.arplant.52.1.725

Goulao, L. F., Santos, J., de Sousa, I., & Oliveira, C. M. (2007). Patterns of enzymatic activity of cell wall-modifying enzymes during growth and ripening of apples. *Postharvest Biology and Technology*, *43*(3), 307–318. http://doi.org/10.1016/j.postharvbio.2006.10.002

Gwanpua, S. G., Mellidfou, I., Boeckx, J., Kyomugasho, C., Bessemans, N., Verlinden, B. E., … Geeraerd, A. H. (2016). Expression analysis of candidate cell wall-related genes associated with changes in pectin biochemistry during postharvest apple softening. *Postharvest Biology and Technology*, *112*, 176–185. http://doi.org/10.1016/j.postharvbio.2015.09.034

Gwanpua, S. G., Van Buggenhout, S., Verlinden, B. E., Christiaens, S., Shpigelman, A., Vicent, V., … Geeraerd, A. (2014). Pectin modifications and the role of pectin-degrading enzymes during postharvest softening of Jonagold apples. *Food Chemistry*, *158*, 283–291. http://doi.org/10.1016/j.foodchem.2014.02.138

Gwanpua, S. G., Verlinden, B. E., Hertog, M. L. A. T. M., Van Impe, J., Nicolai, B. M., &

Geeraerd, A. H. (2013). Towards flexible management of postharvest variation in fruit firmness of three apple cultivars. *Postharvest Biology and Technology*, *85*, 18–29. http://doi.org/10.1016/j.postharvbio.2013.04.015

Harker, F. R., Stec, M. G. H., Hallett, I. C., & Bennett, C. L. (1997). Texture of parenchymatous plant tissue: a comparison between tensile and other instrumental and sensory measurements of tissue strength and juiciness. *Postharvest Biology and Technology*, *11*(2), 63–72. http://doi.org/10.1016/S0925-5214(97)00018-5

Houben, K., Jolie, R. P., Fraeye, I., Van Loey, A. M., & Hendrickx, M. E. (2011). Comparative study of the cell wall composition of broccoli, carrot, and tomato: structural characterization of the extractable pectins and hemicelluloses. *Carbohydrate Research*, *346*(9), 1105–1111. http://doi.org/10.1016/j.carres.2011.04.014

Jackman, R. L., Marangoni, A. G., & Stanley, D. W. (1992). The effects of turgor pressure on puncture and viscoelastic properties of tomato tissue. *Journal of Texture Studies*, *23*(4), 491–505. http://doi.org/10.1111/j.1745-4603.1992.tb00036.x

Kyomugasho, C., Christiaens, S., Shpigelman, A., Van Loey, A. M., & Hendrickx, M. E. (2015). FT-IR spectroscopy, a reliable method for routine analysis of the degree of methylesterification of pectin in different fruit- and vegetable-based matrices. *Food Chemistry*, *176*, 82–90. http://doi.org/10.1016/j.foodchem.2014.12.033

Longhi, S., Hamblin, M. T., Trainotti, L., Peace, C. P., Velasco, R., & Costa, F. (2013). A candidate gene based approach validates *Md-PG1* as the main responsible for a QTL impacting fruit texture in apple (*Malus* x *domestica* Borkh). *BMC Plant Biology*, *13*, 37. http://doi.org/10.1186/1471-2229-13-37

Ly-Nguyen, B., Van Loey, A. M., Fachin, D., Verlent, I., & Hendrickx, M. E. (2002). Partial purification, characterization, and thermal and high-pressure inactivation of pectin methylesterase from carrots (*Daucus carrota* L.). *Journal of Agricultural and Food Chemistry*, *50*(19), 5437–5444. http://doi.org/10.1021/jf011666v

Markovič, O., & Kohn, R. (1984). Mode of pectin deesterification byTrichoderma reesei pectinesterase. *Experientia*, *40*(8), 842–843. http://doi.org/10.1007/BF01951984

McQueen-Mason, S. J., & Cosgrove, D. J. (1995). Expansin mode of action on cell walls. Analysis of wall hydrolysis, stress relaxation, and binding. *Plant Physiology*, *107*(1), 87–100. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=161171&tool=pmcentrez&rendertype=abstract

Ng, J. K. T., Schröder, R., Brummell, D. A., Sutherland, P. W., Hallett, I. C., Smith, B. G., … Johnston, J. W. (2015). Lower cell wall pectin solubilisation and galactose loss during early fruit development in apple (*Malus* x *domestica*) cultivar "Scifresh" are associated with slower softening rate. *Journal of Plant Physiology*, *176C*, 129–137. http://doi.org/10.1016/j.jplph.2014.12.012

Ng, J. K. T., Schröder, R., Sutherland, P. W., Hallett, I. C., Hall, M. I., Prakash, R., … Johnston, J. W. (2013). Cell wall structures leading to cultivar differences in softening rates develop early during apple (*Malus* x *domestica*) fruit growth. *BMC Plant Biology*, *13*(1), 183. http://doi.org/10.1186/1471-2229-13-183

Peña, M. J., & Carpita, N. C. (2004). Loss of highly branched arabinans and debranching of rhamnogalacturonan I accompany loss of firm texture and cell separation during prolonged storage of apple. *Plant Physiology*, *135*(3), 1305–1313. http://doi.org/10.1104/pp.104.043679

Renard, C. (2005). Variability in cell wall preparations: quantification and comparison of common methods. *Carbohydrate Polymers*, *60*(4), 515–522. http://doi.org/10.1016/j.carbpol.2005.03.002

Saladié, M., Matas, A. J., Isaacson, T., Jenks, M. A., Goodwin, S. M., Niklas, K. J., … Rose, J. K. C. (2007). A reevaluation of the key factors that influence tomato fruit softening and integrity. *Plant Physiology*, *144*(2), 1012–1028. http://doi.org/10.1104/pp.107.097477

Szymanska-Chargot, M., & Zdunek, A. (2013). Use of FT-IR spectra and PCA to the bulk characterization of cell wall residues of fruits and vegetables along a fraction process. *Food Biophysics*, *8*(1), 29–42. http://doi.org/10.1007/s11483-012-9279-7

Tateishi, A., Inoue, H., & Yamaki, S. (2001). Fluctuations in activities of three beta-galactosidase isoforms from ripening avocado(*Persea americana*) fruit and their different activities against its cell wall polysaccharides as substrates. *Engei Gakkai Zasshi*, *70*(5), 586–592. http://doi.org/10.2503/jjshs.70.586

Tateishi, A., Kanayama, Y., & Yamaki, S. (1996). α-l-Arabinofuranosidase from cell walls of Japanese pear fruits. *Phytochemistry*, *42*(2), 295–299. http://doi.org/10.1016/0031-9422(95)00931-0

Tong, C., Krueger, D., Vickers, Z., Bedford, D., Luby, J., El-Shiekh, A., … Ahmadi, H. (1999). Comparison of softening-related changes during storage of `Honeycrisp' apple, its parents, and `Delicious'. *J. Amer. Soc. Hort. Sci.*, *124*(4), 407–415. Retrieved from http://journal.ashspublications.org/content/124/4/407.abstract

Vrijdaghs, A., Flores-Olvera, H., & Smets, E. (2014). Enigmatic floral structures in *Alternanthera*, *Iresine*, and *Tidestromia* (Gomphrenoideae, Amaranthaceae). A developmental homology assessment. *Plant Ecology and Evolution*, *147*(1), 49–66. Retrieved from https://lirias.kuleuven.be/handle/123456789/457823

Wakasa, Y., Kudo, H., Ishikawa, R., Akada, S., Senda, M., Niizeki, M., & Harada, T. (2006). Low expression of an endopolygalacturonase gene in apple fruit with long-term storage potential. *Postharvest Biology and Technology*, *39*(2), 193–198.

Yoshioka, H., Aoba, K., & Kashimura, Y. (1992). Molecular weight and degree of methoxylation in cell wall polyuronide during softening in pear and apple fruit. *J. Amer. Soc. Hort. Sci.*, *117*(4), 600–606. Retrieved from http://journal.ashspublications.org/content/117/4/600.abstract

Figure Captions

Figure 1. Firmness loss (A), ethylene production (B) and weight loss (C) of 'Kanzi' and 'Golden Delicious' apples during ripening at 18 °C under regular air for 6 weeks. The error bars are the standard error of the mean of 10 fruit for firmness, 6 for ethylene production, and 20 for weight loss measurements. The insert in (A) shows the percentage loss in firmness.

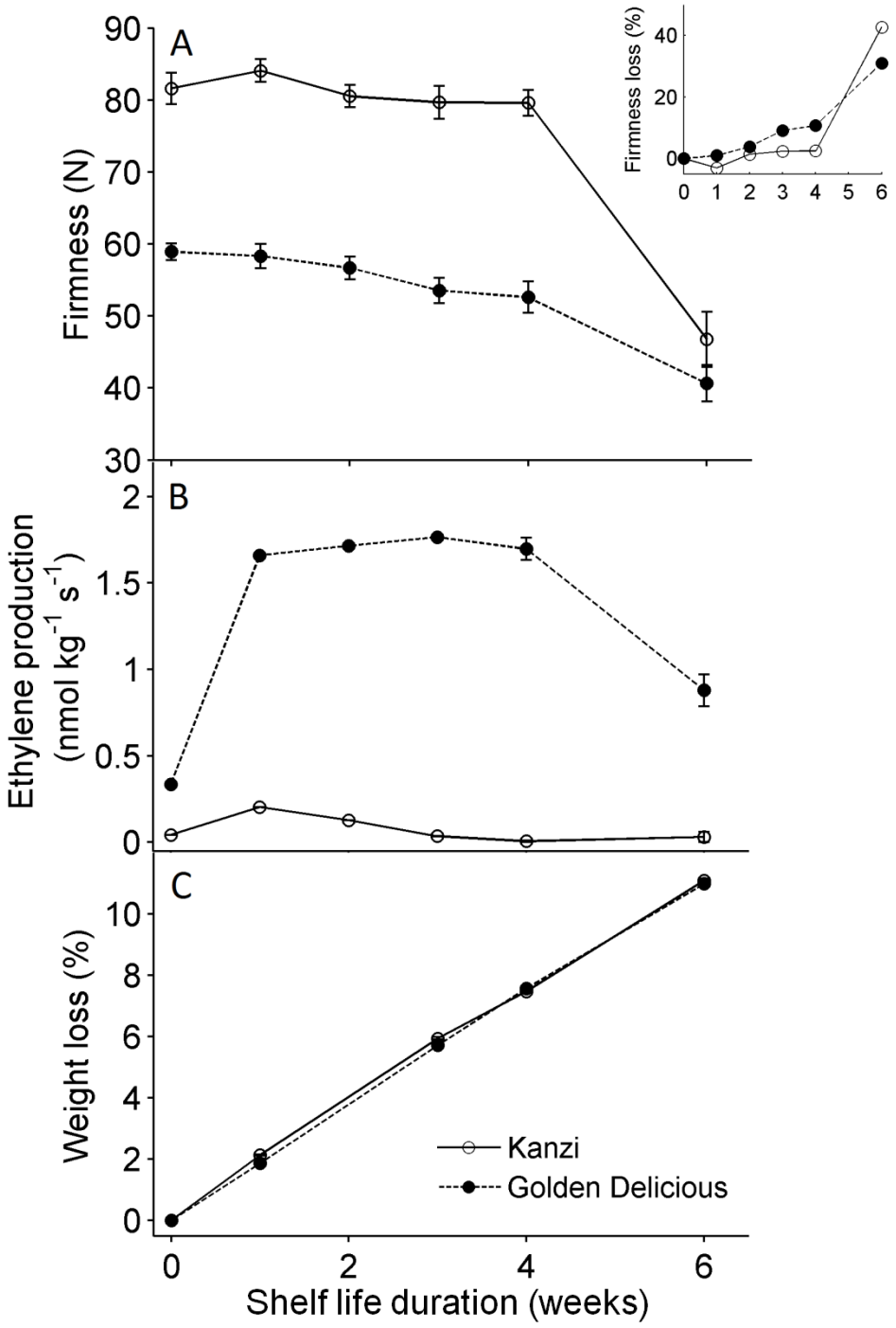
Figure 2. Changes in pectin fractions and depolymerisation of water extractable pectin (WEP) for 'Kanzi' and 'Golden Delicious' apples at the 'onset', 'ripe', and 'senescent' stages. (A) shows the uronic acid content of the WEP, chelator extractable pectin (CEP), and Na_2CO_3 extractable pectin (NEP), with error bars representing the standard error of the mean of four biological replicates (means with the same letters are not significantly different at 5 % significance level). (B) shows size exclusion chromatographic (SEC) profile of the WEP. The SEC profiles are based on the integrated average refractive index (RI) signals of four biological replicate measurements

Figure 3. The arabinose (Ara) and galactose (Gal) content of the water extractable pectin (WEP), chelator extractable pectin (CEP), and Na_2CO_3 extractable pectin (NEP) for fruit at the 'onset', 'ripe', and 'senescent' stages (A). The extend of branching, defined as the ratio of the sum of the Ara and Gal to the rhamnose (Rha) content is shown in (B), while the activities of alpha-arabinofuranosidase (α-AF) and beta-galactosidase (β-GAL) for both 'Kanzi' and 'Golden Delicious' apples are shown in (C). One unit (U) of enzyme activity was defined as the amount of enzyme that hydrolyses the release of 1 μmol of p–nitrophenol per min. The error bars are the standard error for the mean of four biological replicates. Means with the same letters are not significantly different at 5 % significance level.

Figure 4. The degree of methylesterification of the alcohol soluble residues (AIR) (A) and the water extractable pectin(WEP) (B) for 'Kanzi' and 'Golden Delicious' apples at 'onset', 'ripe', and 'senescent' stages. The pectin methylesterase (PME) activity is shown in C. One unit (U) of PME activity was defined as the amount of enzyme required to release 1 μmol of carboxyl group per min, under the assay conditions. The error bars are the standard error for the mean of four biological replicates. Means with the same letters are not significantly different at 5 % significance level.

576 Figure 5. Scanning electron micrograms showing representative tissue fracture patterns during
577 different ripening stages. A-C shows the tissue fracture pattern for fruit at the 'onset', 'ripe', and
578 'senescent' stages, respectively, for 'Kanzi' apples. D-F shows the tissue fracture pattern for fruit
579 at the 'onset', 'ripe', and 'senescent' stages, respectively, for 'Golden Delicious' apples.

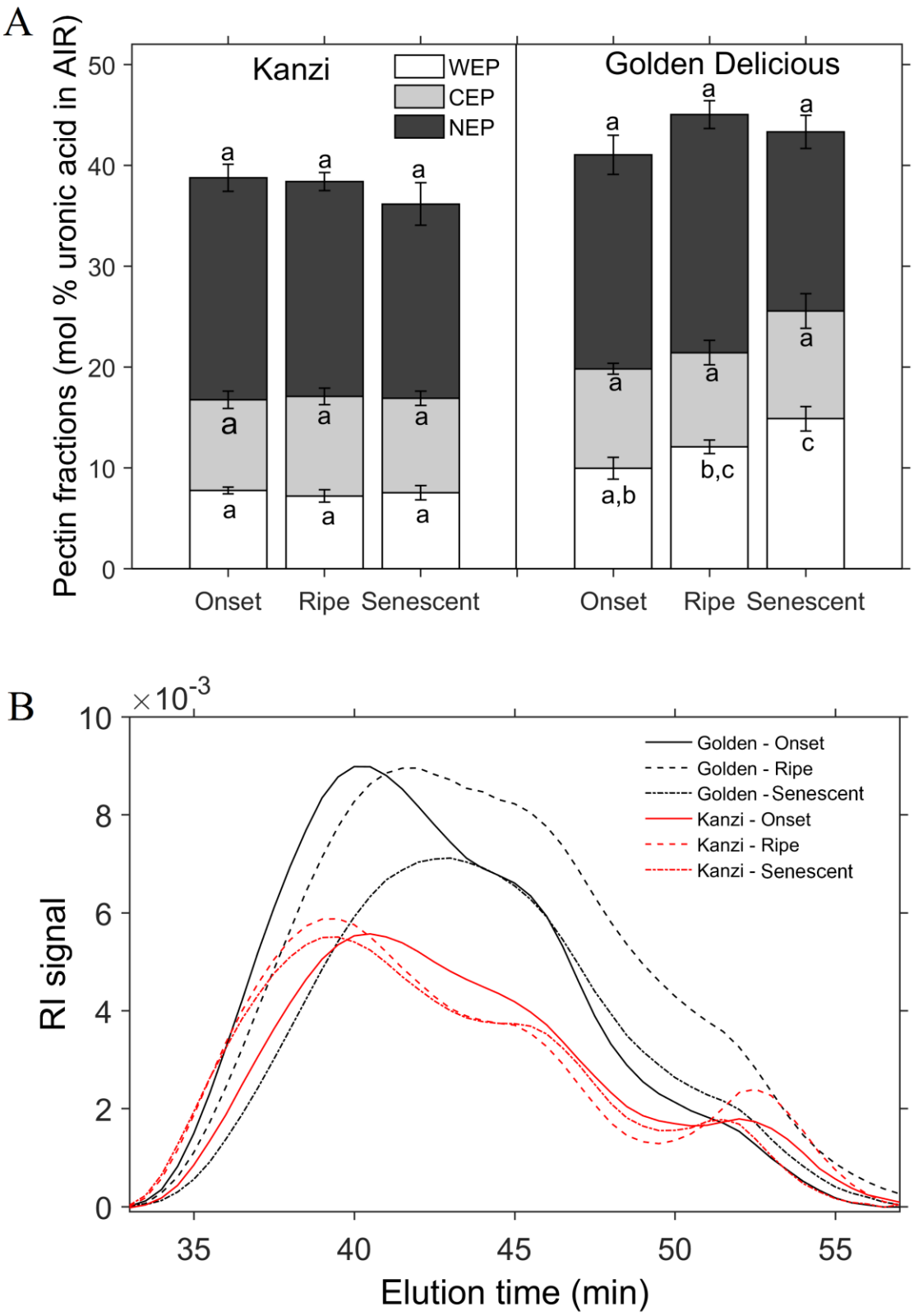
580 Figure 6. Tissue biomechanical properties of 'Kanzi' and 'Golden Delicious' apples at the
581 'onset', 'ripe', and 'senescent' stages. A shows the yield stress, while B shows the yield strain.
582 The error bars in G and H are the standard error for the mean of eight to ten biological replicates.
583 Means with the same letters are not significantly different at 5 % significance level.



584

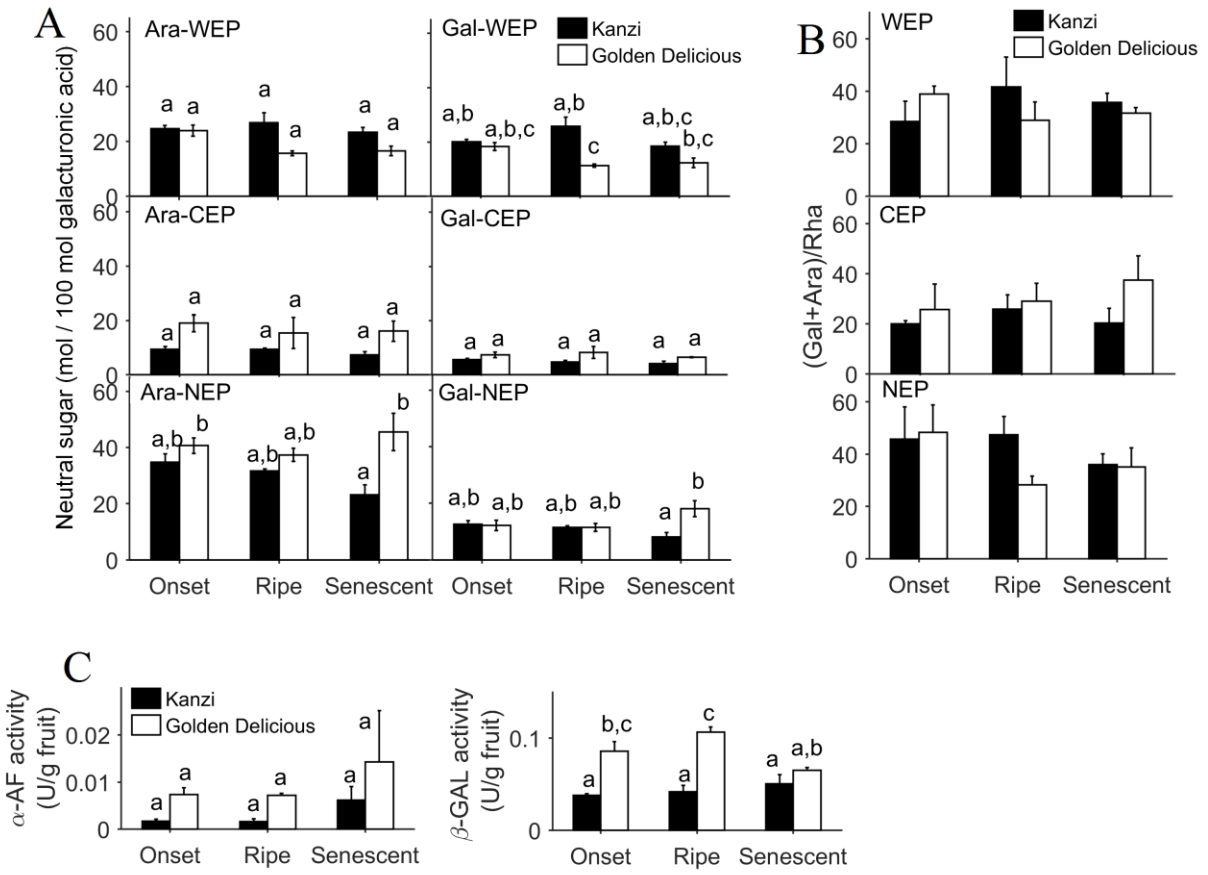
585 Figure 1.

586



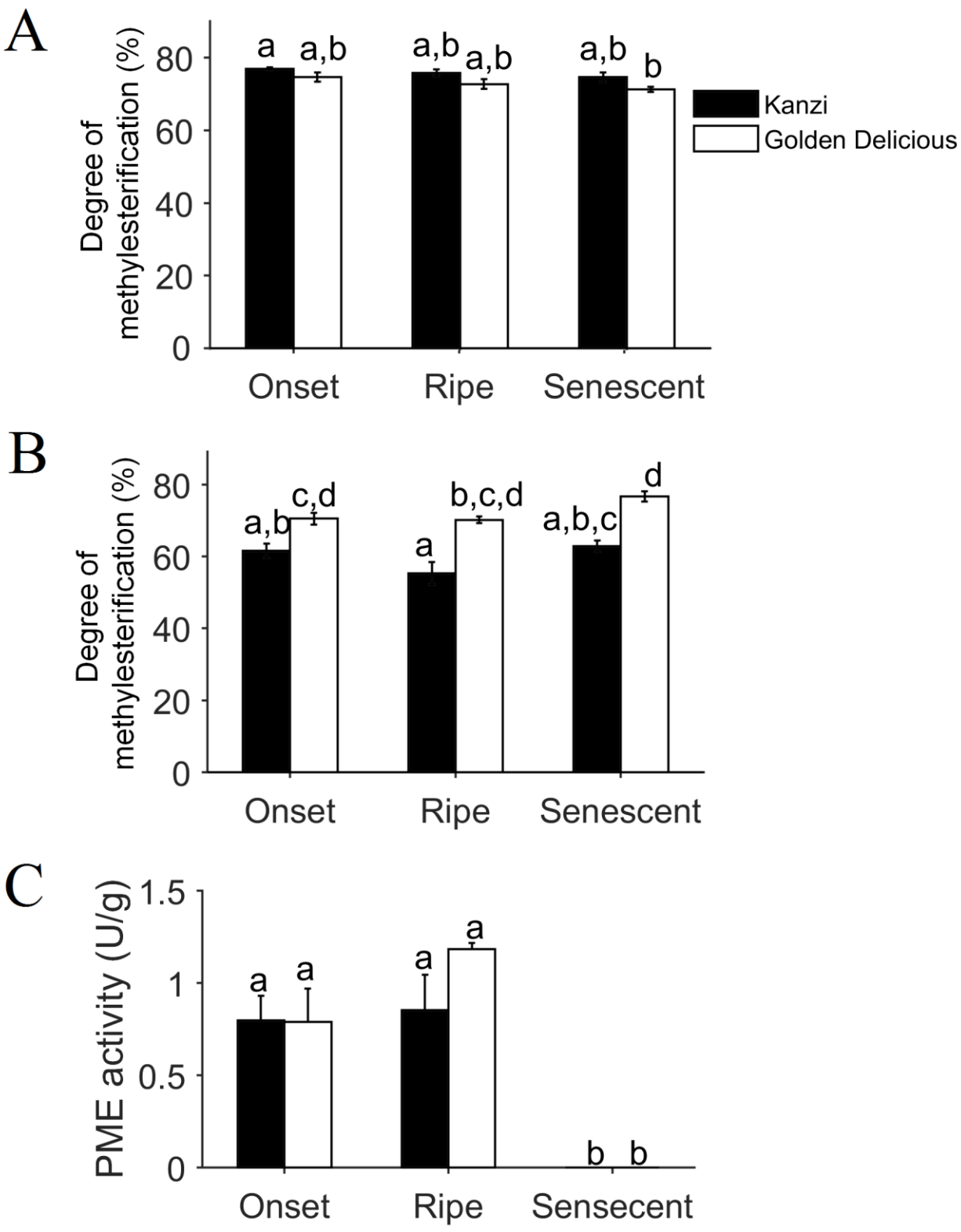
587

588 Figure 2



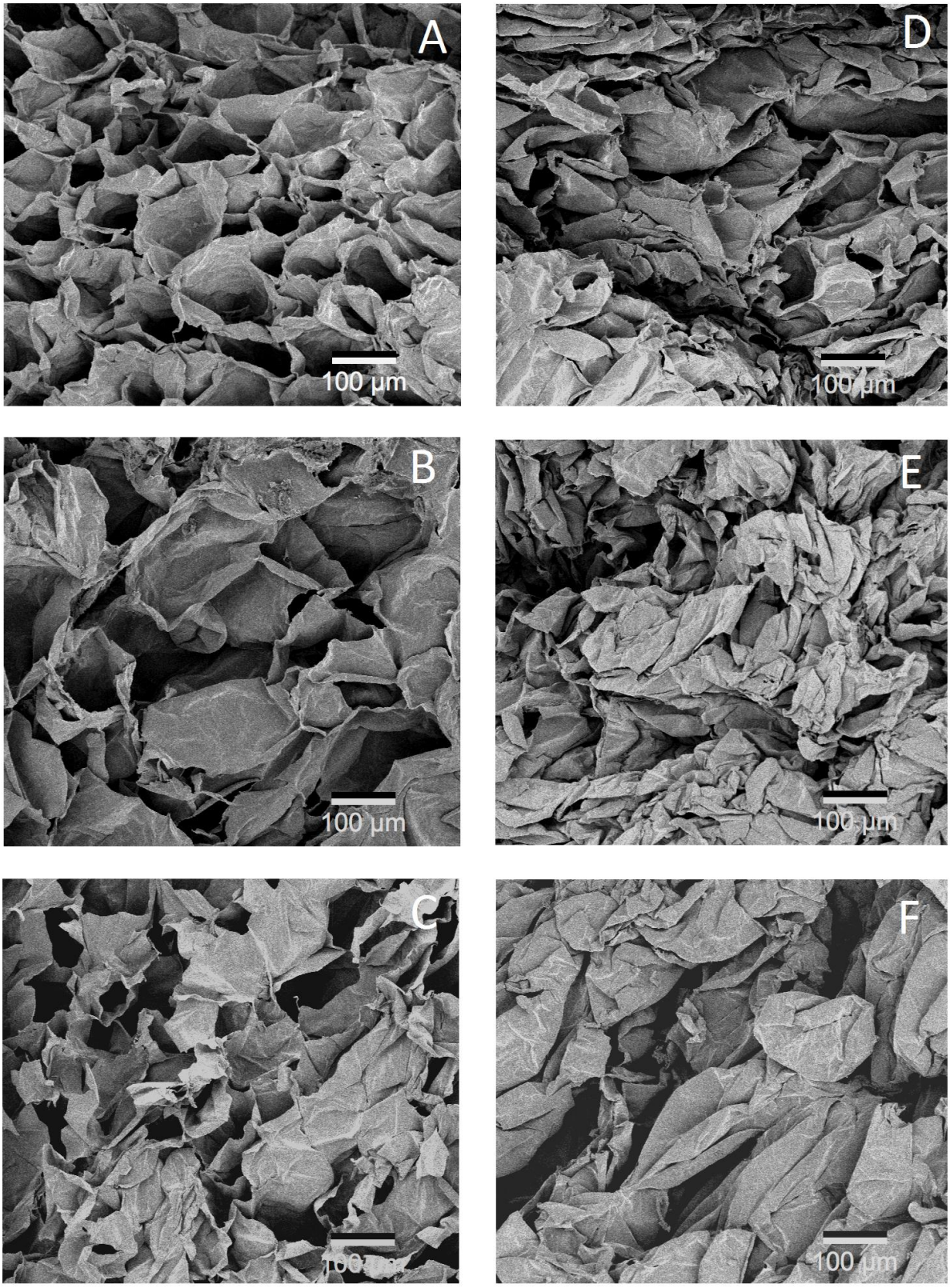
589

590 Figure 3



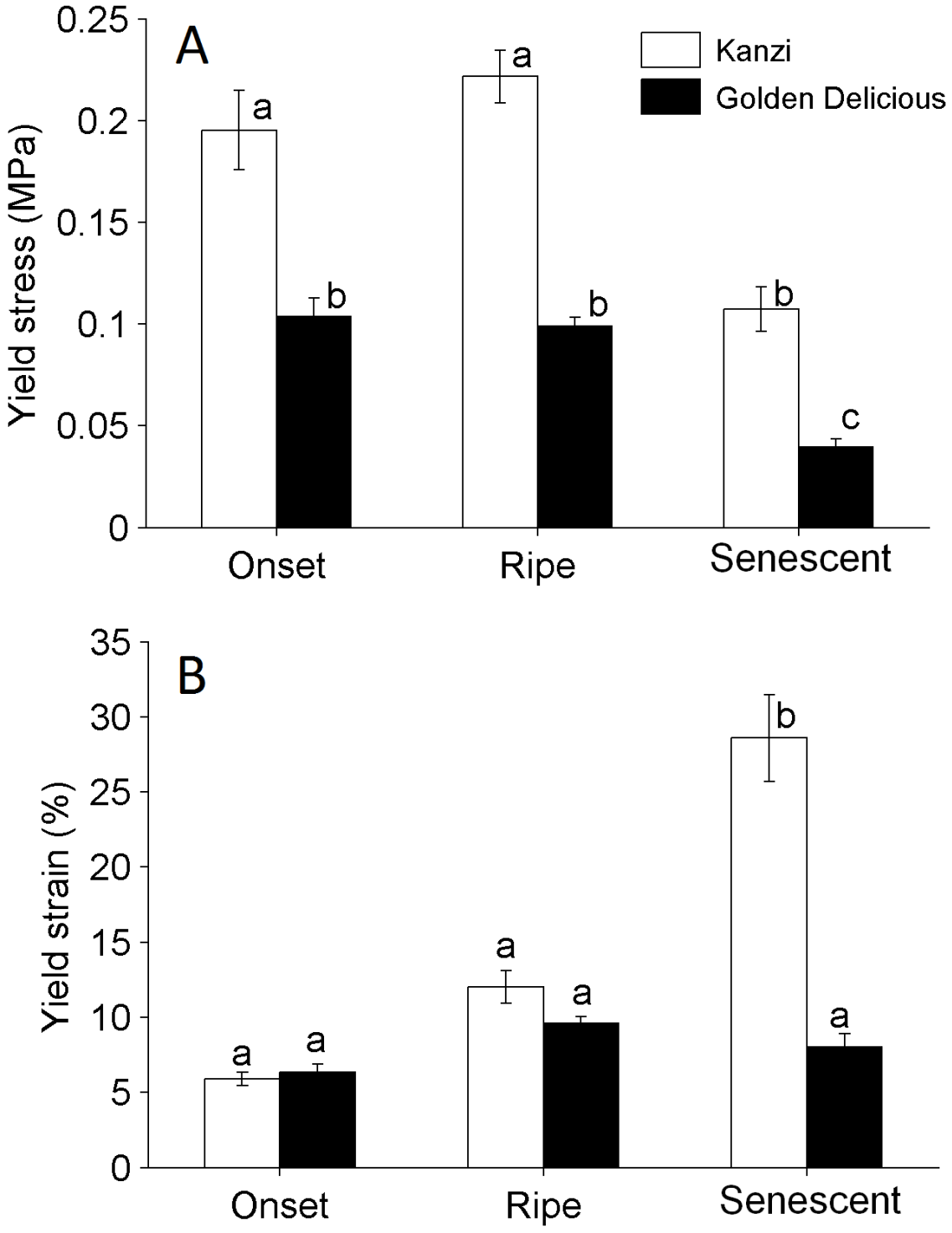
591

592 Figure 4



593

594 Figure 5



595

596 Figure 6